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Processing of soybean hulls to enhance the distribution and extraction of value-added proteins†‡

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Abstract: Soybean hulls contain peroxidase (SBP) and Bowman–Birk type protease inhibitor (BBI). SBP is used in the European bread-baking industry, and BBI isolated from dehulled, defatted soy flour possesses cancer-preventive and anticarcinogenic properties. Because hulls possess a low percentage of nitrogen, with fibre and other carbohydrates being the major components, our objective was to determine whether air classification of milled hulls could be used to fractionate and enrich these value-added proteins. Hulls were pin milled three times at 18 000 rpm, followed by air classification to yield fractions with particle size distributions ranging from <15 to >30 μm. The finely ground fractions with particle sizes ranging from <15 to 18 μm were enriched with nitrogen and lipid when compared with the more coarsely ground fractions. Yields of extractable nitrogenous components from aqueous extracts were quantified at a variety of pHs, homogenisation speeds and times. BBI and SBP were confirmed by polyacrylamide gel electrophoresis with known standards. Based on assays for SBP and BBI activities, BBI, though concentrated in the finer fractions, also appeared in the highest-mass, coarsest fraction, whereas SBP resided mainly in the coarsest fraction. Dry milling and air classification of soybean hulls proved beneficial to distribute SBP into a product stream that facilitates its isolation and purification.
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Keywords: soybean hulls; milling; air classification; aqueous extraction; Bowman–Birk-type inhibitor; peroxidase

INTRODUCTION

Both traditional and potential users of agricultural commodities can be served by investigations that focus on new knowledge of the commodities and new methods for handling them. Soybean hulls, with a US annual production in excess of 9.1×10^8 kg at 6.6 cents kg^{-1}, are an underutilised co-product of the soybean-processing industry. At present, soy hulls are used primarily as a fibre source for dairy and beef cattle, in reduced fat diets for pets and as a bulk additive to feeds. Aspinall *et al*[1] estimated that soy hulls contain about 11% protein, 9–11% galactomannans, 10–12% acidic polysaccharides, 9–10% xylan hemicellulose and about 40% cellulose, with the remainder probably lignin. Soy hull proteins have been investigated for almost two decades, but only four proteins, namely peroxidase (SBP), a hydroxyproline-rich glycoprotein termed extensin, a glycine-rich protein and, most recently, a Bowman-Birk type protease inhibitor (BBI), have been identified.[2–7]

SBP with a p*I* of 4.1 and an apparent molecular mass of 37 kDa is a glycoprotein from soybean hulls.[3] Peroxidases have been used in the bread-baking industry,[8,9] are cross-linking agents for proteins[10] and are oxidative coupling agents for producing phenolic resins.[11] BBI is a serine protease inhibitor which, when isolated and purified from soybean cotyledon, possesses a p*I* of 4.2,[12] has 71 amino acid residues and a molecular mass of 7850 Da and simultaneously inhibits both trypsin and chymotrypsin.[13] BBIs from soybean cotyledon are known cancer-preventive[14] and anticarcinogenic[15] agents. With an efficient processing system and appropriate volumes the cost of manufacturing SBP and BBI from soy hulls would be highly competitive. SBP can replace potassium bromate in the baking industry, formaldehyde as a cross-linking agent in the chemical industry, and horseradish peroxidase in the medical diagnostics industry. BBIs could be a potential replacement for the synthetic serine protease inhibitors now used as anticarcinogenic agents.

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Because SBP and BBI represent minor constituents of soybean hull, fundamental departures from established processing methods will be required to devise commercial processes that would give these proteins a competitive advantage in the marketplace. Traditional processing ignores the benefits of natural segregation of specialised polymeric structures and bioactive constituents. Plant components are dispersed, randomised and even transformed deleteriously under extreme processing conditions. Expedient measures that ensure volume conversions of seed components to carbohydrate, protein and oil, for example, waste otherwise useful structural elements and bioactivities that should be considered part of the inherent value of the commodity. The approach to be used incorporates two principal thrusts: (1) technology for the systematic and economical disassembly of soy hulls using selective air classification;[16] (2) use of processing techniques to concentrate the proteins free of the coextracted carbohydrate components.

Our objectives for this investigation were to optimise the conditions for the aqueous extraction of nitrogenous components from previously prepared air-classified soy hull fractions[14] and to concentrate and confirm the identity of SBP and BBI in those extracts.

MATERIALS AND METHODS

Materials

Three different commercial sources of cold-processed soybean hulls designated A, B and C were investigated for nitrogen extractability and SBP activity. The proximate analysis of these hulls, performed by University of Missouri, Experimental Station Chem Lab, Columbia, MO, USA, is given in Table 1.

Speciality chemicals and electrophoretic and chromatographic materials were obtained as follows: *N*-glutaryl-L-phenylalanine-*p*-nitroanilide (GPNA); Coomassie Brilliant Blue R-250; α-chymotrypsin, type VII from bovine pancreas; trypsin–chymotrypsin inhibitor (Bowman–Birk inhibitor) from soybean; bovine albumin, fraction V; pyrogallol; hemicellulase from *Aspergillus niger* (EC 232-799-9), 1.58 units mg^{-1} solid; soybean peroxidase (EC 1.11.1.7), 5000 units; SP Sephadex C-25 cation exchange resin from Sigma (St Louis, MO, USA); acrylamide, methylene bisacrylamide and sodium dodecyl sulphate (SDS) broad-range molecular weight standards from Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals used in this study were of reagent grade.

Table 1. Proximate analysis (g kg^{-1}, dry matter basis) of soybean hulls from different commercial sources designated A, B and C

Component	A	B	C
Kjeldahl nitrogen	20.9	23.3	31.6
Crude fat	29.3	32.9	41.0
Crude fibre	372.8	336.6	328.9
Ash	55.4	47.6	46.6

Extraction protocols for source A soybean hulls

Source A hulls were finely ground in an S.500 disc mill (Glen Mills Inc, Clifton, NJ, USA) and defatted batchwise with diethyl ether at a hull/solvent ratio of 1:10 (w/v). Each batch for three successive extractions was intermittently stirred for 30 min prior to decanting the extractables. The solids, air dried, were divided equally into 15 g batches. Individual batches were placed in 20 oz Mason jars to which 375 ml of water was added. The endogenous pH of the suspension while stirring with a magnetic stirrer was measured. Upon removal of the stir bar the Mason jar was capped with the chamber cover sealed with a teflon cover plug, and the 20 mm diameter × 150 mm length sawtooth bottom generator was inserted through the cover hole 8.75 in from the generator head of a PRO 300D benchtop homogeniser (PRO Scientific Inc, Monroe, CT, USA). The suspension, cooled in an ice bath, was homogenised for 15 min at 15 000 rpm. The homogenate was centrifuged at 15 344 × *g* for 30 min at 23 °C. The supernatant was filtered under vacuum in a Buchner funnel fitted with Whatman 54 filter paper. The filtrate was freeze-dried and the extracted solid residues were oven dried and discarded. Effects of pH, homogenisation speed and time were evaluated for the extent of nitrogen extractability. Hull sources B and C were each ground, defatted and extracted with water under the optimum conditions for nitrogen extractability established for source A hulls. Each of the three freeze-dried extracts was evaluated for peroxidase activity (see 'Analyses'), and the source with the highest activity was then subjected to milling air classification.

Milling and air classification

Soy hulls, source B, weighing 2 kg were ground in a pin mill (model 160Z, Alpine American Corp, Natick, MA, USA) at 18 000 rpm. After two additional regrindings the hulls were fractionated by particle size in a laboratory-scale air classifier (Pillsbury, Minneapolis, MN, USA) as described by Wolf *et al*.[16] Fractions of size <15, 15–18, 19–24, 25–30 and >30 μm were collected. Each fraction was defatted with diethyl ether and extracted with water as described previously.

Analyses

Micro-Kjeldahl nitrogen (KN), ash and moisture contents were determined by AACC methods.[17]

Peroxidase activity was assayed by measuring the maximum linear rate from the increase in absorbance at 420 nm due to the oxidation of pyrogallol to purpurogallin in the presence of hydrogen peroxide. The methodology used was an updated version (cf Sigma Prod No P-1432, 05/26/94) of the original method by Chance and Maehly.[18]

Chymotrypsin inhibitor (CTI) activity was assayed and quantified according to Erlanger *et al*,[19] using GPNA as substrate. CTI activity (CTIA) in mg g^{-1} sample or mg g^{-1} protein is relative to the CTIA

of commercially available, pure BBI run with each evaluation. For CTIA determination, duplicate runs with five dilutions, each in a linear range, were used.

To identify SBP and BBI, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed along with SBP and BBI standards according to the procedure outlined by Sessa and Wolf.[7]

IR spectra were measured on an FTS 6000 FTIR spectrometer (Digilab, Cambridge, CT, USA) equipped with a DTGS (ie deuterated triglycine sulphate) detector. Absorbance spectra of samples on compressed KBr pellets were acquired at $4\,cm^{-1}$ resolution, signal averaged over 32 scans and baseline corrected.

Concentration and identification of SBP from source B air-classified fractions

The crude aqueous extracts of each of the milled, air-classified fractions from source B hulls were subjected to SDS-PAGE with commercial SBP as standard. The air-classified, crude aqueous extract that visually showed the best defined protein band with a mobility equivalent to commercial SBP as standard as well as the highest SBP activity when assayed with pyrogallol (see 'Analyses') was subjected to enzymic breakdown of carbohydrate components with a combination of hemicellulase and pectinase, followed by ultrafiltration/diafiltration on a Pall (Pall Filtron Corp, Northborough, MA, USA) Centramate tangential flow device equipped with a 5 kDa molecular weight cut-off (MWCO) membrane.

Specifically, 10 g of freeze-dried aqueous extract from the air-classified hull fraction with the highest peroxidase activity (ie 5.56 units mg^{-1} protein) was dispersed in 300 ml of 0.2 M acetate buffer (pH 4.0) along with 1 ml of pectinase (Pectinex 3XL from *Aspergillus niger*, EC 3.2.1.15; Sigma). and 1 g of hemicellulase (1.58 units mg^{-1} solid). This dispersion was incubated for 3 h in a shaking water bath at 37 °C. To quench the reaction, the enzyme dispersion was heat inactivated at 80 °C for 15 min. After cooling in an ice bath, the mixture was centrifuged at $15\,000 \times g$ for 30 min at 23 °C. The supernatant was filtered under vacuum in a Buchner funnel through Whatman 54 filter paper.

Discontinuous diafiltration was performed at 25 °C on the filtrate by adding water to give a total volume of 2.5 l. When 2 l of the permeate had been collected at a filtrate flux rate of $2.7\,l\,h^{-1}$ ($0.093\,m^2$), a second 2 l of water was added to the retentate. A third 2 l of water was added to the retentate to complete the diafiltration. The concentrated retentate, consisting of about 175 ml of retentate and one 100 ml rinse, was freeze-dried to give a concentrate, termed SBP concentrate, which was subjected to SDS-PAGE and peroxidase activity analyses.

Concentration and identification of BBI from source B air-classified fractions

CTI activities of aqueous extracts from each of the air-classified fractions from source B hulls were measured. The hull fraction that possessed the highest CTIA was then subjected to the CM Sephadex C-25 column chromatographic procedure of Sessa and Wolf[7] with the following modification. A 5 g batch of freeze-dried aqueous extract from the air-classified fraction with the highest CTIA was dissolved in 90 ml of 0.05 M NaH_2PO_4 buffer (pH 6.5) containing 0.01% sodium azide as preservative. This solution was dialysed for 48 h against 0.01 M sodium acetate buffer (pH 4.0) with 1 kDa MWCO dialysis tubing. The retentate was centrifuged at $15\,344 \times g$ for 15 min at 23 °C. The centrifugate was chromatographed on a 26 mm inner diameter × 100 cm length column (Amersham Biosciences, Piscataway, NJ, USA) packed to a bed height of 90 cm with SP rather than CM Sephadex C-25 cation exchange resin in 0.01 M sodium acetate buffer (pH 4.0) containing 0.01% sodium azide. The elution programme was initiated with the acetate buffer with a collection volume of 10.2 ml per tube at a rate of two tubes per hour. Column eluates were monitored for UV absorbance at 280 nm and for conductivity. When unbound 280 nm-absorbing components reached the baseline, a linear salt gradient consisting of 500 ml of 0.01 M acetate buffer and 500 ml of 0.01 M acetate buffer with 1 M NaCl generated through interconnected cylindrical chambers was initiated.

RESULTS AND DISCUSSION

Nitrogen extractability of soy hulls

As shown in Table 2, the protein content in the extractables dropped from 0.39 to 0.21 g from endogenous pH 6.4 to 3.5, then increased to 0.44 g

Table 2. Effect of pH on nitrogen extractability from source A soybean hulls via homogenisation[a]

pH	Weight of extractables (g, DM)	KN ($g\,kg^{-1}$, DM)	Extract protein content (g)[b]
6.4	1.77	34.5	0.39
5.5	1.70	31.6	0.34
5.0	1.75	27.8	0.30
4.5	1.81	25.5	0.29
4.0	1.92	21.7	0.27
3.5	1.94	18.3	0.21
3.2	2.08	17.2	0.23
3.0	2.31	17.8	0.25
2.5	2.73	24.9	0.44

DM, dry matter basis; KN, Kjeldahl nitrogen. Values are mean of duplicate determinations.

[a] Homogenisation performed with a PRO 300D homogeniser equipped with a sawtooth generator: 15 g sample/375 ml water, 15 000 rpm, 15 min; pH adjusted downward with 6 M HCl and/or 1 M HCl from endogenous pH 6.4.

[b] Extract protein content = KN × 6.25 × extract weight $\times 10^{-3}$.

upon further decrease in pH to 2.5. FTIR spectra of extracts at pH 6.4 and 3.5 were compared to determine the nature of the carbohydrates coextracted with the proteins. In Fig 1(a) the FTIR spectrum of extractables at pH 6.4 is typical of protein with amide I and II absorbances at 1658 and 1527 cm^{-1} respectively, except for the absorbance at 1076 cm^{-1} which is due to carbohydrate C–O–C deformation. Soy hulls contain soluble polysaccharides as well as pectinaceous substances which are extractable under acidic conditions.[20–22] The FTIR spectrum of soy hull extract at pH 3.5 (Fig 1(b)) is indicative of carbohydrate with absorbances at 1094 cm^{-1} due to CO and OCH deformation; 1276 cm^{-1}, C–O–C of ester; shoulder at ~1414cm^{-1}, symmetrical stretch of carboxylate; 1653 and 1740 cm^{-1} from free and esterified carboxyl groups; and 2935 cm^{-1}, CH stretching of CH_3 groups. In Fig 1(b), amide I and II bands are absent; absorbance bands at 1653 and 1740 cm^{-1} are indicative of soy pectin.[23] When two polymers of opposite net charge are mixed, as in the case with anionic polysaccharides at or above their pK value and proteins below their isoelectric point (eg SBP is 4.1[3] and BBI is 4.2[12]), the enthalpy of mixing is exothermic and the charge–charge interactions will lead to complex formation.[24] Though speculative at this point, the increased weight of extractables and extracted protein at pH 2.5 (see Table 2) may well be due to a protein–polysaccharide complex. Pectinaceous substances from soy hulls soaked at pH 2.4 followed by extraction at pH 1.8 and 90 °C contained 7.9% protein.[20] To limit the amount of pectin and other anionic polysaccharides that could complex with SBP and BBI, endogenous pH 6.4 was selected.

Evaluation of the effect of homogenisation speed ranging from 10 000 to 15 000 rpm, including runs at 12 500 and 13 500 rpm, for 15 min at pH 6.4 followed by re-extraction of the solids recovered by centrifugation showed that extraction and re-extraction at 15 000 rpm gave higher yields of solids (ie 2.33 g of extract and 0.51 g of extractable protein from 15 g of hulls) than did all other extractions at the slower speeds, which for extraction at 10 000 rpm amounted to 1.80 g of extract and 0.45 g of extractable protein. At 15 000 rpm we observed better compaction of solids with centrifugation, thereby making recovery of the centrifugate easier upon decanting the liquid from the solid residue. Homogenisation speeds greater than 15 000 rpm caused excessive foaming of the sample.

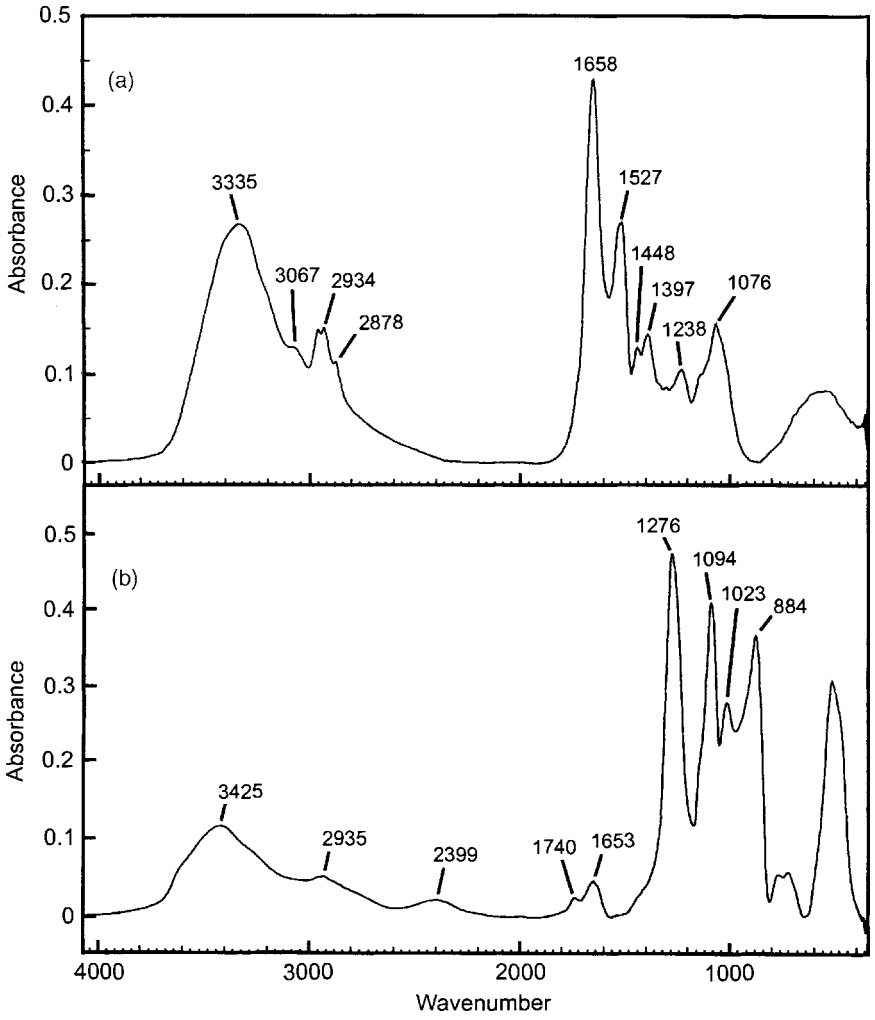

Figure 1. FTIR spectra of freeze-dried aqueous extracts of ground source A soy hulls at (a) pH 6.4 and (b) pH 3.5.

Homogenisation times of 15–120 min at 15 min intervals, when applied at 15 000 rpm and endogenous pH 6.4, were evaluated for optimising nitrogen extractability from soy hulls. Over the range of eight homogenisation times the mean weight of extractables was 1.87 ± 0.18 g and the mean Kjeldahl nitrogen content was 33.7 ± 1.8 g kg^{-1}. Selection of the 15 min extraction time was based on the fact that both the weight of extractables and nitrogen extractability did not differ significantly from the 15 min extraction time results at pH 6.4 as shown in Table 2.

Distribution of soy hull proteins with air classification

Having established the pH, homogenisation speed and time parameters, the next step was to determine which of the three commercial soy hull sources would be used for the extraction of peroxidase. Each of the soy hull sources A, B and C was defatted and extracted as described previously. The freeze-dried extracts representing aqueous extracts from each source, when assayed for peroxidase activity, showed that soy hull source B had an activity of 2.94 units mg^{-1} protein, whereas source A had about one-third of that activity and source C had only trace activity. According to Buttery and Buzzell,[25] SBP activity varies with variety, where high or low activity is highly heritable and is not an extranuclear maternal characteristic. On the other hand, CTIA was present in similar amounts in aqueous extracts from all three hull sources. Based on these findings, source B hulls were used for evaluating the distribution of SBP and CTI active components upon fractionation of the ground hulls by air classification as well as the identity of SBP and CTI active components.

Soy hulls from source B were pin-milled and air-classified into five fractions—1, <15 μm; 2, 15–18 μm; 3, 19–24 μm; 4, 25–30 μm; 5, >30 μm—as reported by Wolf *et al*.[16] The proximate analysis of the milled hulls and the five air-classified fractions is given in Table 3. Nitrogenous components and lipids are mainly distributed in the finer fractions. In the investigation by Wolf *et al*,[16] scanning electron microscopy revealed that fractions 1 and 2 consisted mainly of parenchymal cells, which represent the innermost portion of the hull; fraction 3 contained mainly hour-glass cells, typically found in the middle layer of the hulls, plus parenchyma cell materials; and fractions 4 and 5 included hour-glass cells and clumps of palisade cells (outer cellular layer) adhering to each other.

Each of the samples in Table 3 was defatted with diethyl ether and the defatted solids were then extracted with water according to the protocol established above for nitrogen extractability of soy hulls. The proximate analysis of the aqueous extracts is given in Table 4. The Table 4 nitrogen extractability data complement the solid nitrogen content data in Table 3. In both instances, nitrogenous components are distributed in the more finely air-classified hull fractions. The weight of extractables and nitrogen extractability of pin-milled source B soy hulls were higher than those of source A soy hulls, whose extract weight was 1.77 g and Kjeldahl nitrogen 34.5 g kg^{-1}. Source B hulls possessed 11.5% higher Kjeldahl nitrogen than did source A hulls (see Table 1). The nitrogen extracted from milled source B hulls was 1.9 times greater than that from source A hulls. Source C hulls possessed the highest Kjeldahl nitrogen and crude fat contents of all three sources. The higher nitrogen and fat contents in source C hulls can be attributed to 'soy cotyledon dust', based on electrophoretic evidence for both 7S and 11S storage proteins when aqueous extracts of source C hulls were subjected to SDS-PAGE (data not shown). Higher nitrogen extractability will reflect a difference in hull compositions from the different commercial sources.

Assay of the aqueous extracts in Table 4 for peroxidase activity and CTIA as shown in Table 5 demonstrated that the peroxidase resides mainly in the coarsest fraction 5, whereas the CTIA based on mg g^{-1} protein resides in the finer fractions, with the least amount in the coarsest fraction.

The cellular location of peroxidase is conflicting. Gillikin and Graham[3] report its presence in both the hour-glass and palisade cells, whereas Gijzen *et al*[4] present evidence that it resides primarily in the hour-glass cells. Our findings favour those of Gillikin and Graham.[3] Scanning electron micrographs as reported

Table 3. Proximate analysis (dry matter basis) of milled source B soybean hulls fractionated by air classification

Fraction	Particle size (μm)	Weight recovered (g)	KN (g kg^{-1})	Fat (g kg^{-1})	Ash (g kg^{-1})
Milled hulls[a]	Unsized	—	23.3	32.9	47.6
1	<15	32.7	69.3	113.3	49.6
2	15–18	52.1	68.4	114.4	59.4
3	19–24	327.3	44.4	70.6	61.1
4	25–30	83.2	42.1	66.1	53.7
5	>30	1128.2	18.6	25.2	42.6

KN, Kjeldahl nitrogen.
[a] Alpine milled three times at 18 000 rpm.

Table 4. Proximate analysis (dry matter basis) of freeze-dried aqueous extracts of defatted, milled source B soybean hulls fractionated by air classification

Fraction[a]	Particle size (μm)	Weight of extract (g)	KN (g kg^{-1})	Extract protein content (g)
Milled hulls	Unsized	2.00	57.2	0.72
1	<15	3.95	79.4	1.96
2	15–18	3.61	87.6	1.98
3	19–24	3.34	64.6	1.35
4	25–30	1.95	61.6	0.75
5	>30	1.16	34.4	0.25

KN, Kjeldahl nitrogen.
[a] Homogenisation of samples performed with a PRO 300D homogeniser: 15 g sample/375 ml water, 15 000 rpm, 15 min, endogenous pH 6.4.

Table 5. Soybean peroxidase (SBP) and chymotrypsin inhibitor (CTI) activity assays of freeze-dried aqueous extracts from milled (three times at 18 000 rpm), defatted, air-classified soybean hulls (source B)

Fraction	Particle size (μm)	SBP activity (units mg^{-1} protein)	CTI activity ($mg\,g^{-1}$ protein)	CTI activity ($mg\,g^{-1}$ sample)
Milled control	Unsized	2.78 ± 0.22	125.6	860.1 ± 18.7
1	<15	0.15 ± 0.02	302.6	698.9 ± 13.4
2	15–18	0.27 ± 0.04	301.6	704.6 ± 22.6
3	19–24	1.71 ± 0.02	215.5	780.8 ± 18.6
4	25–30	2.08 ± 0.08	215.1	818.0 ± 24.8
5	>30	5.56 ± 0.39	66.9	576.5 ± 13.9

Values are the means of three replicates ± standard deviation.

by Wolf *et al*[16] of our air-classified fraction 5 showed a predominance of palisade cells in that fraction, where peroxidase activity is highest, while in fraction 3 with a predominance of hour-glass cells the peroxidase activity is about 26.5% of that in fraction 5. On the other hand, CTIA does not appear to be associated with any particular cellular constituent, because its activity is evident in all the fractions. Based on our findings in Table 5, milling and air classification of soy hulls benefit the isolation and purification of peroxidase.

Identification of SBP and BBI in soy hull extracts

CTI active components in the aqueous extracts from each of the air-classified fractions gave similar 280 nm absorbance elution patterns to those observed by Sessa and Wolf[7] when the extracts were chromatographed on SP Sephadex C-25 columns eluted with a linear gradient from zero to 1 M NaCl. Test tube eluates having 280 nm absorbance at molar salt concentrations ranging from 0.21 to 0.80 M were pooled. The pooled eluates (805.8 ml) were concentrated to 175 ml (ie hold-up volume) using a Pall Centramate tangential flow ultrafiltration device equipped with a 5 kDa MWCO polyethersulphone membrane (0.093 m^2), operating at 25 °C and a filtrate flux rate of 2.31 h^{-1}. The retentates were each dialysed against distilled water with 1 kDa MWCO dialysis casings for 48 h under refrigeration to remove buffer salts as well as sodium chloride from the gradient elution. Retentates were each freeze-dried. In this current investigation we used SP rather than CM Sephadex C-25 for our column medium. With this stronger cation exchange resin and the pH used in this study, an increased amount of sample could be chromatographed, yet no change in elution pattern was observed.

The results of our ultrafiltration/discontinuous diafiltration process to generate an SBP concentrate from the coarsest air-classified fraction (ie >30 μm) gave us an approximate recovered weight of 89.8% based on original mass, where the weight of the freeze-dried first permeate was 5.27 g (dry basis) and the combined weight of the freeze-dried second and third permeates was 1.95 g; the weight of the freeze-dried

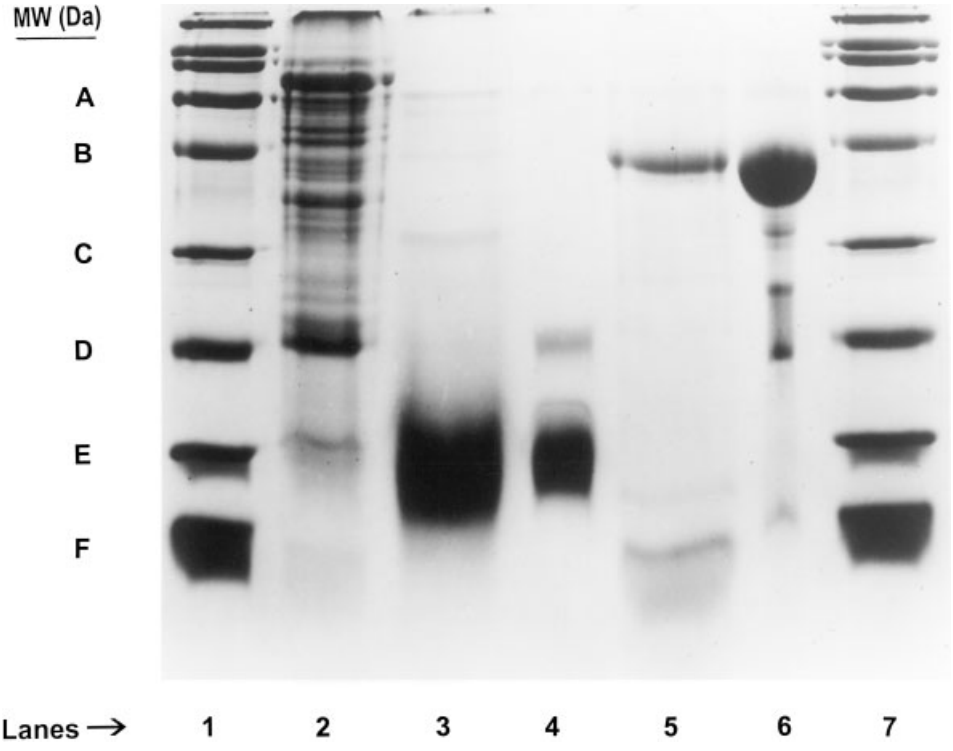

Figure 2. SDS-PAGE of soy hull Bowman–Birk inhibitor (BBI) concentrated by fractionation on SP Sephadex C-25 column and soybean peroxidase (SBP) concentrated by ultrafiltration/diafiltration, both from aqueous extracts of air-classified source B soy hulls, where BBI was isolated from fraction 15–18 μm and SBP from fraction >30 μm. Lanes 1 and 7, molecular weight standards: A, 66 kDa; B, 45 kDa; C, 31 kDa; D, 21 kDa; E, 14 kDa; F, 6 kDa. Lane 2, crude aqueous extract of soy hull; lane 3, soy hull BBI concentrate; lane 4, BBI standard; lane 5, soy hull SBP concentrate; lane 6, SBP standard.

Table 6. Soybean peroxidase (SBP) and chymotrypsin inhibitor (CTI) activities before and after their respective concentration processing from source B soy hulls

Sample	SBP activity (units mg^{-1} protein)	CTI activity ($mg\,g^{-1}$ sample)
BBI	—	3272
SBP	49.2	—
Crude extracts[a]	5.6	721
Purified extracts[b]	23.8	2262

[a] BBI, Bowman–Birk inhibitor. SBP from aqueous extracts of air-classified fraction >30 μm; BBI from fraction <15 μm.
[b] SBP concentrated by subjecting enzymically hydrolysed aqueous extracts to ultrafiltration/diafiltration; BBI concentrated by cation exchange column chromatography.

retentate was 1.76 g. These recovered weights did not include the weight of the centrifugation pellet or the weight of the buffer salt.

Peroxidase assay of the retentate gave 23.82 units mg^{-1} protein, whereas the original extract had 5.56 units mg^{-1} protein. Thus discontinuous diafiltration increased the peroxidase activity 4.28-fold.

Table 6 summarises the SBP and CTI activities for the CTI concentrate from the aqueous extract of the air-classified finest fraction (<15 μm) subjected to column chromatography on SP Sephadex C-25 and for the SBP concentrate from the aqueous extract of the air-classified coarsest fraction (>30 μm) subjected to ultrafiltration/discontinuous diafiltration. Based on our commercial SBP standard with 49.2 units mg^{-1} protein, our SBP concentrate possesses 48.4% of the activity of the standard. Comparing the CTIA of our BBI isolated from the air-classified finest fraction (ie <15 μm) with that of the commercial standard, our preparation has 69.1% of the CTIA of the standard. The methodologies used in this investigation are effective for concentrating both SBP and BBI.

SDS-PAGE was used to identify SBP and BBI in the concentrates. As can be observed in Fig 2, lanes 1 and 7 represent the protein molecular weight standards; lane 2 is from an aqueous extract of ground source B soy hulls; lane 3 is the BBI concentrate from column chromatography of the aqueous extract from the air-classified finest fraction (ie <15 μm), which compares favourably with the mobility of the BBI standard in lane 4; the SBP concentrate from the aqueous extract of the air-classified coarsest fraction (ie >30 μm) subjected to ultrafiltration/diafiltration in lane 5 has a mobility identical to that of the commercial SBP standard.

CONCLUSION

The peroxidase activity of soy hulls is species-specific, whereas BBI was found in similar amounts in all three sources investigated. Air classification of ground soy hulls proved effective for distributing the SBP into the highest-mass, coarsest fraction; this process in combination with enzyme treatment of extracts subjected to ultrafiltration/diafiltration facilitated its isolation and purification. BBI can be effectively concentrated from aqueous extracts of ground soy hulls by cation exchange column chromatography.

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